MycoKeys

MycoKeys 112: 17–34 (2025)
DOI: 10.3897/mycokeys.112.140487

Research Article

# Two new species of *Xylaria* (Xylariaceae, Ascomycota) associated with fallen leaves in Hainan Tropical Rainforest National Park, China

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## Abstract

*Xylaria* is a large and complex genus of macrofungi, playing a critical role in ecosystems as decomposers and possessing antimicrobial, anticancer, and antioxidant properties. This paper described two new *Xylaria* species discovered on fallen leaves in Hainan Tropical Rainforest National Park, based on morphological characteristics and genetic sequences. Detailed color illustrations and comparisons with similar species were provided. The main characteristics of *X. acifer* included long, needle-shaped stromata, ellipsoid to fusoid ascospores, and prominent non-cellular appendages. The stromata of *X. tuberculosa* had an aggregated, knobby fertile part, with smaller ascospores also featuring non-cellular appendages. Additionally, a key to 46 *Xylaria* species associated with fallen leaves and petioles worldwide was established.

**Key words:** Foliicolous, molecular phylogenetics, morphology, *Xylaria*, taxonomy

OPEN ACCESS

This article is part of:
***Diversity, taxonomy, and systematics of macrofungi from tropical Asia***
*Edited by Olivier Raspé, Rui-Lin Zhao, Jennifer Luangsa-ard*

Academic editor:
Jennifer Luangsa-ard
**Received:** 29 October 2024
**Accepted:** 6 December 2024
**Published:** 7 January 2025

**Citation:** Pan X, Chen Z, Chen Y, Lei J, Chen X, Wu T, Li Y (2025) Two new species of *Xylaria* (Xylariaceae, Ascomycota) associated with fallen leaves in Hainan Tropical Rainforest National Park, China. MycoKeys 112: 17–34. https://doi.org/10.3897/mycokeys.112.140487

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## Introduction

*Xylaria* Hill ex Schrank, established in 1789, is the type genus of the Xylariaceae family, with *X. hypoxylon* (L.) Grev. as the type species. Most *Xylaria* species are saprotrophic, a few are parasitic, and some form symbiotic relationships with plants as endophytes (Martín 1970; Rogers 1979; Deshmukh et al. 2022). These fungi are widely distributed across tropical, subtropical, and temperate regions (Ma et al. 2022) and are commonly referred to as "dead man's fingers" (Deshmukh et al. 2022). *Xylaria* species exhibit unique survival adaptations, and their melanin production creates a physical and chemical barrier that other competing fungi cannot penetrate, allowing them to expel excess water in extremely humid environments and thrive in harsh conditions (Rajtar et al. 2023).

To date, *Xylaria* species have been reported on six of the seven continents (Dennis 1956, 1958; Greenhalgh and Chesters 1968; Rogers and Samuels 1986; Whalley 1996; Ju and Rogers 1999; Rogers 2000; San Martín et al. 2001). Most known *Xylaria* species grow on wood, while a minority are found on fallen leaves and petioles, fallen fruits and seeds, and termite nests and soil (Rogers et al. 2005; Ju and Hsieh 2007; Ju et al. 2018; Fournier et al. 2020; Wangsawat et al. 2021; Hsieh et al. 2022; Ju et al. 2022; Ju and Hsieh

2023). Fallen leaves and petioles are important substrates for *Xylaria* species, but these species are often difficult to detect due to their fragile, small, and sparse stromata (Ju and Hsieh 2023; Pan et al. 2024). It is also common for different species stromata to grow on the same leaf, complicating the study of *Xylaria* species associated with these substrates (Ju and Hsieh 2023; Pan et al. 2024).

Another challenge in identifying *Xylaria* species associated with fallen leaves and petioles arises from *X. filiformis* (Alb. & Schwein.) Fr. Studies on *Xylaria* species related to fallen leaves and petioles worldwide often reference *X. filiformis* (Ju and Hsieh 2023), but the identification of this species is rather problematic. *Xylaria filiformis*, originally described as *Sphaeria filiformis* Alb. & Schwein. in 1805, has subsequently been classified as *Hypoxylon filiforme* (Alb. & Schwein.) Rabenh., *Xylosphaera filiformis* (Alb. & Schwein.) Dennis, and *Podosordaria filiformis* (Alb. & Schwein.) P.M.D. Martin (http://www.indexfungorum.org/, 29 October 2024). Ju and Hsieh (2023) clarified the identity of *X. filiformis*, systematically categorizing 42 species associated with fallen leaves and petioles and making significant contributions to the taxonomy of this group. Pan et al. (2024) further expanded this group by describing *X. diaoluoshanensis* Xiao Y. Pan and *X. fulvotomentosa* Xiao Y. Pan, increasing the number of species to 44. In this paper, we continued to explore this substrate group by describing two new species, *X. acifer* and *X. tuberculosa*, based on morphological characteristics and molecular sequences. Additionally, we provide a key to 46 *Xylaria* species associated with fallen leaves and petioles.

## Materials and methods

### Specimen source and morphological study

The specimens were collected from the Diaoluoshan Area of Hainan Tropical Rainforest National Park and are currently stored at the Forest Resources Institute of Hainan Academy of Forestry. Habitat photographs of the specimens were taken using a Canon D3 camera (Canon Corporation, Tokyo, Japan) and a Huawei Mate 50 smartphone (Huawei Technologies Co., Ltd., Shenzhen, China). The macroscopic characteristics of the specimens were examined using a VHX-5000 digital microscope (Keyence Corporation, Osaka, Japan). Microscopic morphological features were observed and measured with a fully automatic optical microscope DM6B (Leica Corporation, Wetzlar, Germany). In this study, N represented the number of ascospores observed and measured, and M represented the average size of the ascospores.

### DNA extraction and amplification

Total DNA from the specimens was extracted using the cetyltrimethylammonium bromide (CTAB) Plant Genome Rapid Extraction Kit (Aidlab Biotechnologies, Beijing, China). This DNA was used to amplify gene sequences from three loci: ITS, RPB2, and TUB2. Each PCR reaction was performed in a 40 μL system (16 μL $ddH_2O$, 20 μL HS™ Mix, 1 μL forward primer, 1 μL reverse primer, and 2 μL DNA template). The ITS region was amplified using primers ITS4/

ITS5 (White et al. 1990) with the following PCR program: initial denaturation at 95 °C for 3 minutes, followed by 30 cycles of 94 °C for 40 seconds, 55.8 °C for 45 seconds, and 72 °C for 1 minute, with a final extension at 72 °C for 10 minutes (Pan et al. 2022). RPB2 was amplified using primers 7CR/5F (Liu et al. 1999) and RPB2AM-1bf/RPB2AM-7R (Miller and Huhndorf 2005), and TUB2 was amplified using primers T1/T22 (O'Donnell and Cigelnik 1997). The PCR program for both RPB2 and TUB2 was: initial denaturation at 95 °C for 3 minutes, followed by 35 cycles of 94 °C for 1 minute, 52 °C for 1 minute, and 72 °C for 1.5 minutes, with a final extension at 72 °C for 10 minutes (Hsieh et al. 2005). The PCR products were sequenced by Tianyi Huiyuan Gene Technology Co., Ltd. (Wuhan, China). The obtained sequences were submitted to GenBank to receive accession numbers.

### Phylogenetic analysis

In this study, ten newly extracted sequences were analyzed alongside 256 Xylariaceae sequences collected from the National Center for Biotechnology Information (NCBI), resulting in a total of 266 gene sequences (Table 1). These included 100 ITS sequences, 85 TUB2 sequences, and 81 RPB2 sequences. A phylogenetic tree was constructed based on the ITS-TUB2-RPB2 dataset. Sequences were verified and aligned using the online tool MAFFT v.7 (http://mafft.cbrc.jp/alignment/server/), manually trimmed and optimized in BioEdit v. 7.0.5.2 (Hall 1999), and the three loci sequences were concatenated using MEGA v.6.0 (Felsenstein 1981; Tamura et al. 2011). Phylogenetic analysis was conducted using both maximum likelihood (ML) and Bayesian inference (BI) methods. ML analysis was performed in RAxML v.8.2.10 (Stamatakis 2014), while BI analysis was conducted using MrBayes v.3.2.6 (Huelsenbeck and Ronquist 2001; Ronquist et al. 2012; Song et al. 2022). *Poronia pileiformis* (Berk.) Fr. was selected as the outgroup. The phylogenetic tree was viewed and adjusted in FigTree v.1.4.3.

## Results

### Molecular phylogeny

After alignment in MAFFT v.7, the character positions for the ITS, TUB2, and RPB2 sequences were determined to be 822, 2112, and 1255, respectively. After post-trimming, the ITS sequence retained 520 character positions, and TUB2 and RPB2 retained 1525 and 1027, respectively. The concatenated ITS-TUB2-RPB2 sequence dataset thus consisted of 3072 characters, including 1374 parsimony-informative positions. The results of the phylogenetic analyses showed no significant differences between the BI and ML trees. Bayesian posterior probabilities (≥ 0.95) and RAxML bootstrap values (≥ 50%) were marked on the phylogenetic tree (Fig. 1). In the phylogenetic tree, *X. acifer* clustered together with *X. hedyosmicola*, Hai X. Ma & X.Y. Pan, while *X. tuberculosa* was closely related to *X. acifer*, *X. betulicola*, Hai X. Ma, Lar.N. Vassiljeva & Yu Li, *X. crinalis*, Hai X. Ma, Lar.N. Vassiljeva & Yu Li, *X. filiformis*, *X. hedyosmicola*, and *X. simplicissima* (Pers.) Y.M. Ju & H.M. Hsieh.

**Table 1.** List of species, specimens, and gene sequence accession numbers used in this study. New sequences provided in this study are in bold. "NA"= not available.

| Taxon | Substrate/Origin | Specimen No. | GenBank No. | | | Reference |
|---|---|---|---|---|---|---|
| | | | ITS | TUB2 | RPB2 | |
| *Poronia pileiformis* | Cow dung/China | WSP 88113001 | GU324760 | GQ502720 | GQ853037 | Hsieh et al. (2010) |
| ***Xylaria acifer*** | **Fallen leaves/China** | **HAFFR 122** | **PQ483147** | **PQ498329** | **PQ498326** | **This study** |
| ***X. acifer*** | **Fallen leaves/China** | **HAFFR 130** | **PQ483150** | NA | **PQ498328** | **This study** |
| *X. acuminatilongissima* | Termite nests/China | HAST 623 | EU178738 | GQ502711 | GQ853028 | Hsieh et al. (2010) |
| *X. aethiopica* | Pods of *Millettia ferruginea*/Ethiopia | YMJ 1136 | MH790445 | MH785221 | MH785222 | Fournier et al. (2018) |
| *X. adscendens* | Wood/Guadeloupe | HAST 570 | GU300101 | GQ487708 | GQ844817 | Hsieh et al. (2010) |
| *X. allantoidea* | Trunk/China | HAST 94042903 | GU324743 | GQ502692 | GQ848356 | Hsieh et al. (2010) |
| *X. amphithele* | Dead leaves/Guadeloupe | HAST 529 | GU300083 | GQ478218 | GQ844796 | Hsieh et al. (2010) |
| *X. apoda* | Bark/China | HAST 90080804 | GU322437 | GQ495930 | GQ844823 | Hsieh et al. (2010) |
| *X. arbuscula* | Bark/China | HAST 89041211 | GU300090 | GQ478226 | GQ844805 | Hsieh et al. (2010) |
| *X. arbuscula* var. *plenofissura* | Wood/China | HAST 93082814 | GU339495 | GQ478225 | GQ844804 | Hsieh et al. (2010) |
| *X. aristata* | Petioles/Indonesia | YMJ 1823 | OQ883719 | NA | NA | Ju and Hsieh (2023) |
| *X. atrodivaricata* | Termite nests/China | HAST 95052001 | EU178739 | GQ502713 | GQ853030 | Hsieh et al. (2010) |
| *X. badia* | Bamboo culm/China | HAST 95070101 | GU322446 | GQ495939 | GQ844833 | Hsieh et al. (2010) |
| *X. bambusicola* | Bamboo culm/Thailand | JDR 162 | GU300088 | GQ478223 | GQ844801 | Hsieh et al. (2010) |
| *X. berteri* | Bark/USA | JDR 256 | GU324750 | GQ502698 | GQ848363 | Hsieh et al. (2010) |
| *X. berteri* | Bark/China | HAST 90112623 | GU324749 | AY951763 | GQ848362 | Hsieh et al. (2010) |
| *X. betulicola* | Leaves of *Betula*/China | FCATAS 750 | MF774332 | NA | NA | Ma and Li (2018) |
| *X. brevifurcata* | Ground/China | YMJ 100062601 | OQ845482 | OQ818432 | OQ851526 | Ju et al. (2023) |
| *X. brunneovinosa* | Termite nests/China | HAST 720 | EU179862 | GQ502706 | GQ853023 | Hsieh et al. (2010) |
| *X. castorea* | Wood/New Zealand | PDD 600 | GU324751 | GQ502703 | GQ853018 | Hsieh et al. (2010) |
| *X. chaiyaphumensis* | Termite nests/Thailand | SWUF16-11.4 | MT622776 | OQ845433 | OQ851585 | Wangsawat et al. (2021); Ju et al. (2023) |
| *X. cirrata* | Termite nests/China | HAST 664 | EU179863 | GQ502707 | GQ853024 | Hsieh et al. (2010) |
| *X. coccophora* | Wood/French | HAST 786 | GU300093 | GQ487701 | GQ844809 | Hsieh et al. (2010) |
| *X. crinalis* | Wood/China | FCATAS 751 | MF774330 | NA | NA | Ma and Li (2018) |
| *X. crozonensis* | Bark/France | HAST 398 | GU324748 | GQ502697 | GQ848361 | Hsieh et al. (2010) |
| *X. cubensis* | Log/Russian Far East | HAST 477 | NA | GQ502699 | GQ848364 | Hsieh et al. (2010) |
| *X. culleniae* | Pod/Thailand | JDR 189 | GU322442 | GQ495935 | GQ844829 | Hsieh et al. (2010) |
| *X. delicatula* | On decaying leaves/French Guiana | GS 2775 | OQ883720 | NA | NA | Ju and Hsieh (2023) |
| *X. diaoluoshanensis* | Fallen leaves/China | HAFFR 115 | OR702611 | OR726655 | NA | Pan et al. (2024) |
| *X. diaoluoshanensis* | Fallen leaves/China | HAFFR 117 | OR702612 | OR726656 | OR757125 | Pan et al. (2024) |
| *X. diaoluoshanensis* | Fallen leaves/China | HAFFR 127 | OR702613 | OR726657 | NA | Pan et al. (2024) |
| *X. fabacearum* | Seed pods of Fabaceae/Thailand | MFLU 16-1061 | NR171104 | MT212220 | MT212202 | Perera et al. (2020) |
| *X. fabaceicola* | Seed pods of Fabaceae/Thailand | MFLU 16-1072 | NR171103 | MT212219 | MT212201 | Perera et al. (2020) |
| *X. feejeensis* | Bark/China | HAST 92092013 | GU322454 | GQ495947 | GQ848336 | Hsieh et al. (2010) |
| *X. ficicola* | Fallen leaves and petioles of *Ficus auriculata*/ China | HMJAU 22818 | MZ351258 | NA | NA | Pan et al. (2022) |
| *X. filiformis* | Herbaceous stem/Iran | GUM 1052 | KP218907 | NA | NA | Hashemi et al. (2015) |
| *X. fimbriata* | Termite nests/French West Indies | HAST 491 | GU324753 | GQ502705 | GQ853022 | Hsieh et al. (2010) |
| *X. fulvotomentosa* | Fallen leaves/China | HAFFR 124 | OR702619 | OR726658 | OR757121 | Pan et al. (2024) |
| *X. fulvotomentosa* | Fallen leaves/China | HAFFR 129 | OR702620 | OR726659 | OR757122 | Pan et al. (2024) |
| *X. furcata* | Termite nests/China | HAST 646 | GU324757 | GQ502715 | GQ853032 | Hsieh et al. (2010) |
| *X. furcatula* | Ground/China | YMJ 98070105 | OQ845483 | OQ818433 | OQ851527 | Ju et al. (2023) |
| *X.* cf. *glebulosa* | Fruit/French West Indies | HAST 431 | GU322462 | GQ495956 | GQ848345 | Hsieh et al. (2010) |
| *X. grammica* | Wood/China | HAST 479 | GU300097 | GQ487704 | GQ844813 | Hsieh et al. (2010) |
| *X. griseosepiacea* | Termite nests/China | HAST 641 | EU179865 | GQ502714 | GQ853031 | Hsieh et al. (2010) |
| *X. hedyosmicola* | Fallen leaves of *Hedyosmum orientale*/China | FCATAS 856 | MZ227121 | MZ221183 | MZ683407 | Pan et al. (2022) |
| *X. hedyosmicola* | Fallen leaves of *Hedyosmum orientale*/China | FCATAS 857 | MZ227023 | MZ221184 | MZ851780 | Pan et al. (2022) |
| *X. hoehnelii* | Ground/China | YMJ 95052006 | OQ845489 | OQ818439 | OQ851533 | Ju et al. (2023) |
| *X. hypoxylon* | Wood/Belgium | HAST 152 | GU300096 | GQ260187 | GQ844812 | Hsieh et al. (2010) |
| *X. hypoxylon* | Wood/China | HAST 95082001 | GU300095 | GQ487703 | GQ844811 | Hsieh et al. (2010) |

| Taxon | Substrate/Origin | Specimen No. | GenBank No. | | | Reference |
|---|---|---|---|---|---|---|
| | | | ITS | TUB2 | RPB2 | |
| *X. ianthinovelutina* | Fruit of *Swietenia*/Martinique | HAST 553 | GU322441 | GQ495934 | GQ844828 | Hsieh et al. (2010) |
| *X. insignifurcata* | Ground/China | YMJ 95071201 | OQ845496 | OQ818446 | OQ851540 | Ju et al. (2023) |
| *X. intraflava* | Termite nests/China | HAST 725 | EU179866 | GQ502718 | GQ853035 | Hsieh et al. (2010) |
| *X. juruensis* | *Arenga engleri*/China | HAST 92042501 | GU322439 | GQ495932 | GQ844825 | Hsieh et al. (2010) |
| *X. laevis* | Wood/Martinique | HAST 419 | GU324746 | GQ502695 | GQ848359 | Hsieh et al. (2010) |
| *X. leavis* | Bark/China | HAST 95072910 | GU324747 | GQ502696 | GQ848360 | Hsieh et al. (2010) |
| *X. lindericola* | Fallen leaves of *Lindera robusta*/China | FCATAS 852 | MZ005635 | MZ031978 | MZ031982 | Pan et al. (2022) |
| *X. lindericola* | Fallen leaves of *Lindera robusta*/China | FCATAS 853 | MZ005636 | MZ031979 | MZ048749 | Pan et al. (2022) |
| *X. liquidambaris* | Fruits of *Liquidambar formosana*/China | HAST 93090701 | GU300094 | GQ487702 | GQ844810 | Hsieh et al. (2010) |
| *X. longissima* | Wood/China | FCATAS 749 | MF774331 | NA | NA | Ma and Li (2018) |
| *X. longissima* | Wood/Iran | IRAN 16582 F | KP218906 | NA | NA | Hashemi et al. (2015) |
| *X. meliacearum* | Petioles and infructescence of *Guarea guidonia*/ Puerto Rico | JDR 148 | GU300084 | GQ478219 | GQ844797 | Hsieh et al. (2010) |
| *X. minuscula* | Fallen leaves of *Castanopsis carlesii* var. *Sessilis*/China | YMJ 90102701 | OQ883721 | NA | NA | Ju and Hsieh (2023) |
| *X. multiplex* | Wood/USA | JDR 259 | GU300099 | GQ487706 | GQ844815 | Hsieh et al. (2010) |
| *X. muscula* | Dead branch/French West | HAST 520 | GU300087 | GQ478222 | GQ844800 | Hsieh et al. (2010) |
| *X. nigripes* | Termite nests/China | HAST 653 | GU324755 | GQ502710 | GQ853027 | Hsieh et al. (2010) |
| *X. oxyacanthae* | Fallen seeds/USA | JDR 859 | GU322434 | GQ495927 | GQ844820 | Hsieh et al. (2010) |
| *X. oxyacanthae* | Fruits/Germany | LZ 2010-502 | HQ414587 | NA | NA | Roensch et al. (2010) |
| *X. palmicola* | Fruits/New Zealand | PDD 604 | GU322436 | GQ495929 | GQ844822 | Hsieh et al. (2010) |
| *X. petchii* | Fallen branches/China | HAFFR 60 | OR702616 | OR735171 | NA | Pan et al. (2024) |
| *X. petchii* | Fallen leaves of *Daphniphyllum paxianum*/China | HAFFR 118 | OR702617 | OR735172 | OR757123 | Pan et al. (2024) |
| *X. petchii* | Fallen leaves of *Daphniphyllum paxianum*/China | HAFFR 126 | OR702618 | OR735173 | OR757124 | Pan et al. (2024) |
| *X. phyllocharis* | Dead leaves/French West | HAST 528 | GU322445 | GQ495938 | GQ844832 | Hsieh et al. (2010) |
| *X. plebeja* | Trunk/China | HAST 91122401 | GU324740 | GQ502689 | GQ848353 | Hsieh et al. (2010) |
| *X. polymorpha* | Wood/USA | JDR 1012 | GU322460 | GQ495954 | GQ848343 | Hsieh et al. (2010) |
| *X. polymorpha* | Stump/Germany | M:M-0125909 | FM164944 | NA | NA | Persoh et al. (2009) |
| *X. polysporicola* | Fallen leaves of *Polyspora hainanensis*/China | FCATAS 848 | MZ005592 | MZ031976 | MZ031980 | Pan et al. (2022) |
| *X. polysporicola* | Fallen leaves of *Polyspora hainanensis*/China | FCATAS 849 | MZ005591 | MZ031977 | MZ031981 | Pan et al. (2022) |
| *X. reevesiae* | Fruits of *Reevesia formosana*/China | HAST 90071609 | GU322435 | GQ495928 | GQ844821 | Hsieh et al. (2010) |
| *X. regalis* | Log of *Ficus racemose*/India | HAST 920 | GU324745 | GQ502694 | GQ848358 | Hsieh et al. (2010) |
| *X. rogersii* | Fruits of *Magnolia* sp./China | FCATAS 915 | MZ648827 | NA | MZ707121 | Ma et al. (2022) |
| *X. schimicola* | Fruits of *Schima noronhae*/China | FCATAS 896 | MZ648850 | MZ695787 | MZ707114 | Ma et al. (2022) |
| *X. schweinitzii* | Bark/China | HAST 92092023 | GU322463 | GQ495957 | GQ848346 | Hsieh et al. (2010) |
| *X. scoparia* | Ground/China | YMJ 97072802 | OQ843005 | OQ818480 | OQ851574 | Ju et al. (2023) |
| *X. siamensis* | Termite nests/Thailand | SWUF 17-20.2 | MT622765 | OQ845437 | OQ851589 | Wangsawat et al. (2021); Ju et al. (2023) |
| *X. sicula* f. *major* | Fallen leaves/China | HAST 90071613 | GU300081 | GQ478216 | GQ844794 | Hsieh et al. (2010) |
| *X. sihanonthii* | Termite nests/Thailand | SWUF 18-1.3 | MT622785 | MW459242 | NA | Wangsawat et al. (2021) |
| *X. simplicissima* | On herbaceous stems/Finland | MP 111004 | OQ883722 | NA | NA | Ju and Hsieh (2023) |
| *X. striata* | Branch/China | HAST 304 | GU300089 | GQ478224 | GQ844803 | Hsieh et al. (2010) |
| *X. subintraflava* | Termite nests/Thailand | SWUF 16-4.3 | MT622762 | OQ845438 | OQ851590 | Wangsawat et al. (2021); Ju et al. (2023) |
| *X. tenellifurcata* | Ground/China | YMJ 98052001 | OQ845498 | OQ818448 | OQ851542 | Ju et al. (2023) |
| *X. theaceicola* | Fruits of *Schima villosa*/China | FCATAS 903 | MZ648848 | MZ695788 | MZ707115 | Ma et al. (2022) |
| *X. thienhirunae* | Termite nests/Thailand | SWUF 16-6.2 | MT622770 | MW459235 | NA | Wangsawat et al. (2021) |
| ***X. tuberculosa*** | **Fallen leaves/China** | **HAFFR 63** | **PQ483149** | **PQ498331** | NA | **This study** |
| ***X. tuberculosa*** | **Fallen leaves/China** | **HAFFR 123** | **PQ483148** | **PQ498330** | **PQ498327** | **This study** |
| *X. venosula* | Twigs/USA | HAST 94080508 | EF026149 | EF025617 | GQ844806 | Hsieh et al. (2010) |
| *X. venustula* | Bark/China | HAST 88113002 | GU300091 | GQ487699 | GQ844807 | Hsieh et al. (2010) |
| *X. vittatipiliformis* | Dead leave/Guadeloupe | CLLGUAD 029 | OQ883723 | NA | NA | Ju and Hsieh (2023) |
| *X. vivantii* | Fruits of *Magnolia* sp./Martinique | HAST 519 | GU322438 | GQ495931 | GQ844824 | Hsieh et al. (2010) |
| *X. wallichii* | Fruits of *Schima wallichii*/China | FCATAS 923 | MZ648861 | MZ695793 | MZ707118 | Ma et al. (2022) |
| *X. xylarioides* | Wood/Iran | GUM 1051 | KP218909 | NA | NA | Hashemi et al. (2015) |

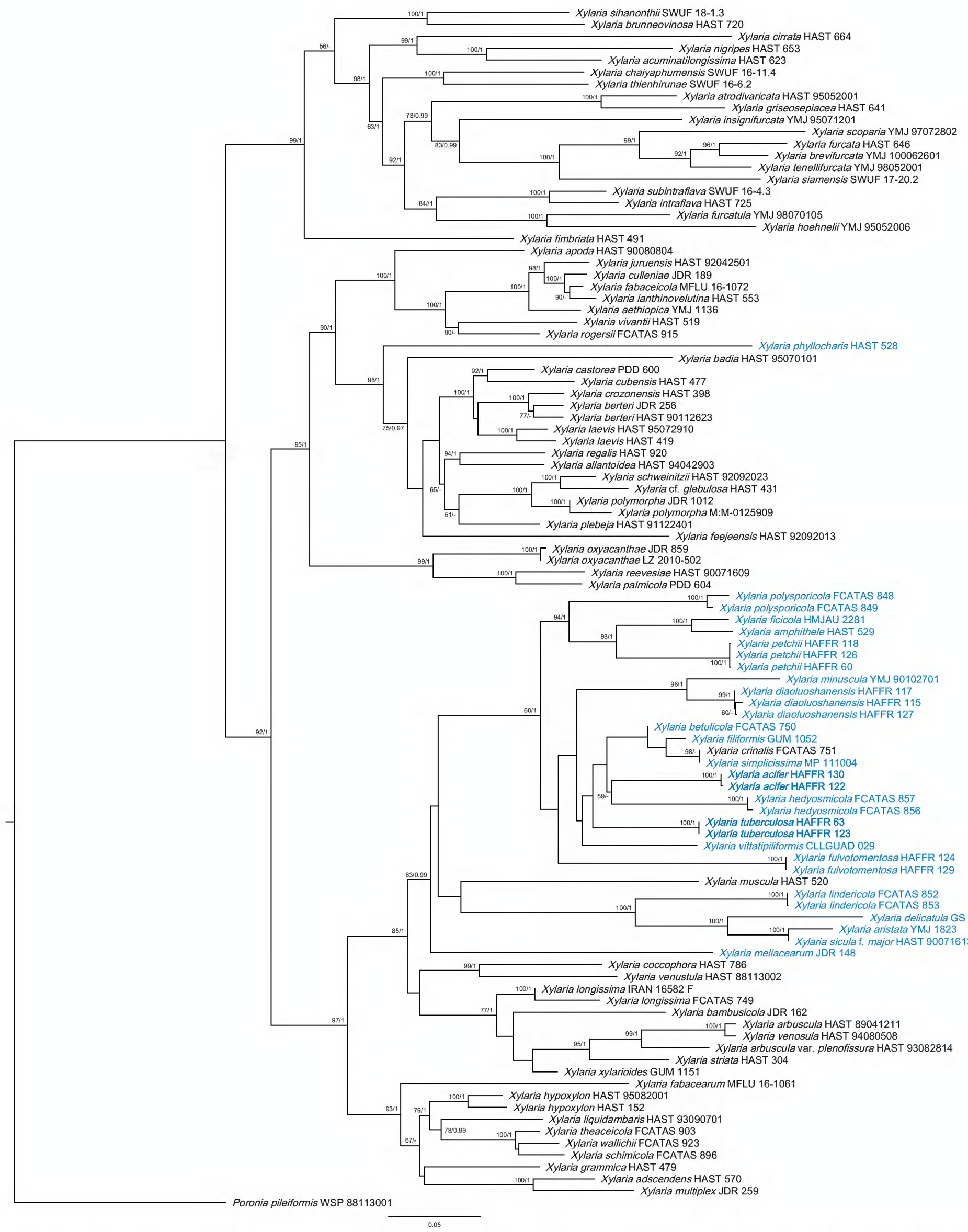


**Figure 1.** Phylogenetic tree of *Xylaria* based on the ITS-TUB2-RPB2 sequence dataset constructed using ML analysis. Support values from ML and BI analyses (bootstrap supports ≥ 50%, posterior probability values ≥ 0.95) are indicated above or below the respective branches (ML/BI). Species associated with fallen leaves and petioles are marked in blue, and the species described in this paper are in bold.

## Taxonomy

***Xylaria acifer* Xiao Y. Pan, sp. nov.**
MycoBank No: 856020
Fig. 2

**Holotype.** China • Hainan Province, Diaoluoshan Area of Hainan Tropical Rainforest National Park; 18°43'35"N, 109°52'11"E; elevation 973 m; on fallen leaves, 18 June 2023, Xiaoyan Pan (HAFFR 122). GenBank accession numbers PQ483147 (ITS), PQ498329 (TUB2), and PQ498326 (RPB2).

**Diagnosis.** Differs from *X. hedyosmicola* by its smaller ascospores. Differs from *X. vittatipiliformis* by the absence of band-like stripes on its stromata surfaces and its smaller ascospores. Differs from *X. vittiformis* by the absence of band-like stripes on its stromata surfaces.

**Etymology.** "*acifer*" refers to the needle-like shape of the stromata.

**Teleomorph.** ***Stromata*** upright, solitary, needle-like, unbranched, 25–50 mm total length; acute sterile apex, 2–10 mm; fertile portion 3–10 mm long × 0.8–1.2 mm diam., elongate cylindrical, composed of tightly arranged perithecia; stipe glabrous, 15–40 mm long × 0.5–1 mm diam., longitudinally striate, the base slightly swollen; surface roughened, black, with conspicuous to half-exposed perithecial mounds; interior white to creamy; texture soft. ***Perithecia*** spherical, 250–500 µm diam. ***Ostioles*** papillate. ***Asci*** with eight ascospores arranged in uniseriate manner, cylindrical, 90–150 µm total length, spore-bearing part 60–85 µm long × 5.5–7.5 µm wide, stipe 30–70 µm long, with a bluing apical ring in Melzer's reagent, tubular to slightly urn-shaped, 1.8–3.5 µm high × 1.4–2.5 µm diam. ***Ascospores*** brown to dark brown, unicellular, ellipsoid to fusiform, inequilateral, with narrowly rounded ends, smooth, (9.3–)10–11(–12) × 3.7–4.7 µm (M = 10.6 × 4.2 µm, N = 40), with straight germ slit nearly the full length of the spore on the flattened side, a hyaline sheath visible in 1% SDS, swollen at both ends, forming non-cellular appendages.

**Remarks.** *Xylaria acifer* clusters with *X. hedyosmicola* in the phylogenetic tree, and they share some similarities in stromatal morphology, yet they also exhibit distinct differences. The stromata of *X. hedyosmicola* possess more prominent perithecial mounds (half-exposed to fully exposed), and its ascospores are brown, ellipsoid, and larger ((12–)13–15(–16.7) × (6–) 6.5–7.5 (–8.5) µm) (Pan et al. 2022). *Xylaria vittatipiliformis* Y.-M. Ju, H.-M. Hsieh & Fournier and *X. vittiformis* Y.-M. Ju & H.-M. Hsieh are morphologically similar to *X. acifer*. *Xylaria vittatipiliformis* can be distinguished by its band-like striped grayish brown outer peeling layer and larger ascospores ((10–)11–12(–12.5) × (5.5–)6–7(–7.5) µm) (Ju and Hsieh 2023). *Xylaria vittiformis* is separated from *X. acifer* by its grayish brown outer peeling layer split into band-like stripes, smaller perithecia (150–200 µm broad), and ellipsoid ascospores, (8–)8.5–10(–11) × (4–)4.5–5(–5.5) µm (Ju and Hsieh 2023).

*Xylaria appendiculatoides* Y.M. Ju & H.M. Hsieh, *X. filiformis*, and *X. simplicissima* are also similar to *X. acifer* in stromatal morphology but are distinctly different. *Xylaria appendiculatoides* has stromata with sharper

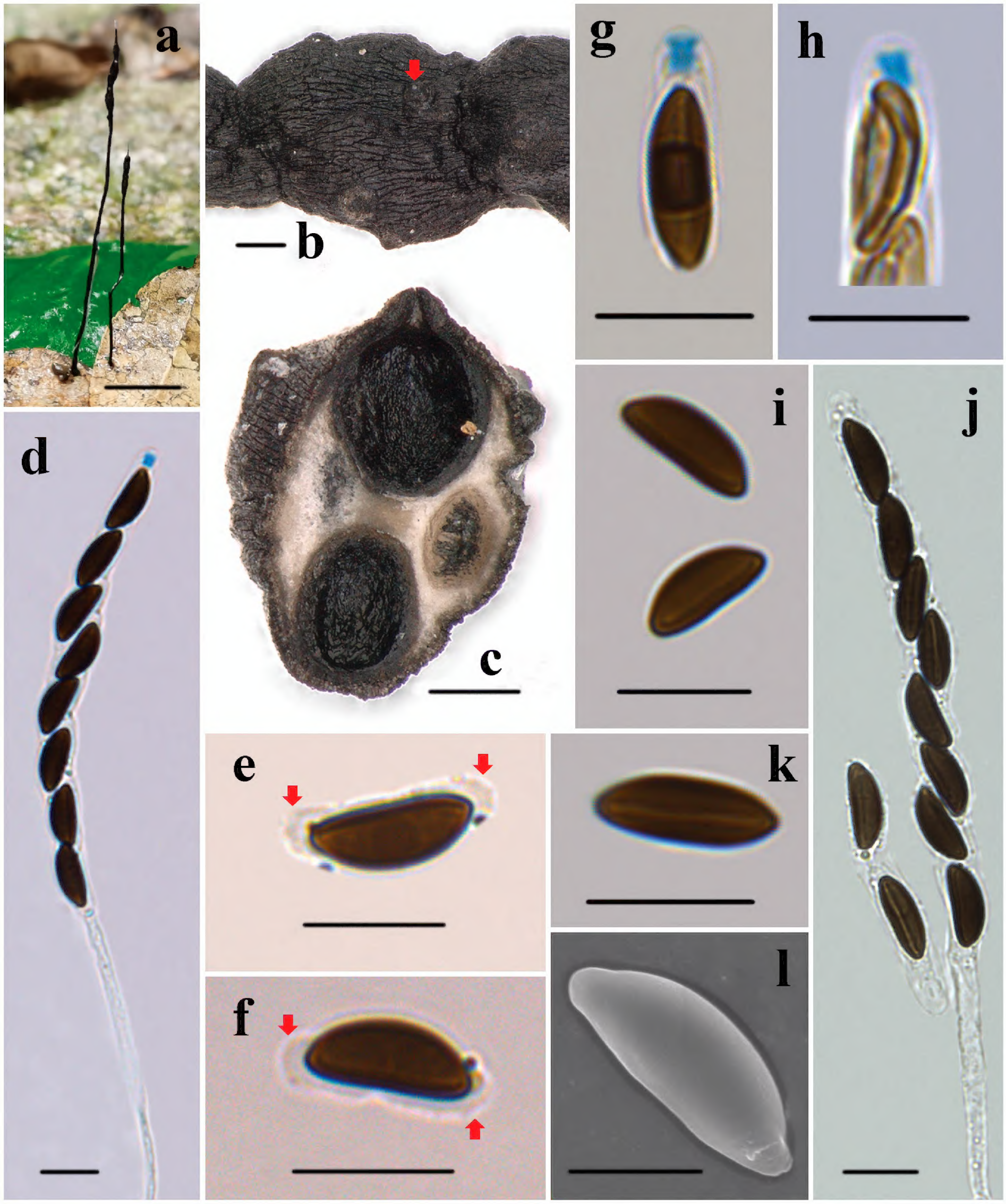


**Figure 2.** *Xylaria acifer* (HAFFR 122) a stromata on leaves **b** stromatal surface and ostioles (arrow) **c** section through stroma, showing perithecia **d** ascus in Melzer's reagent **e, f** ascospore showing non-cellular appendages in 1% SDS (arrows) **g, h** ascal apical ring in Melzer's reagent **i** ascospores in Melzer's reagent **j** ascus in water **k** ascospore showing a nearly spore-length straight germ slit in Melzer's reagent **l** ascospore under SEM. Scale bars: 1 cm (**a**); 200 µm (**b, c**); 10 µm (**d–k**); 5 µm (**l**).

ostioles (conic-papillate, tilting upwards) and larger ascospores ((14–)15–16(–17) × (6.5–)7.0–7.5(–8) µm) (Ju and Hsieh 2023). *Xylaria filiformis* and *X. simplicissima* have ascospores that are markedly different from those of *X. acifer*. *Xylaria filiformis* has light brown ascospores that are larger ((9.5–

)11.5–13.5(–14.5) × (4–)4.5–5.5(–6) µm) (Ju and Hsieh 2023). *Xylaria simplicissima* has larger ascospores ((15–)16.5–19(–21.5) × (5–)5.5–6.5(–7.5) µm) that lack non-cellular appendages (Ju and Hsieh 2023). Additionally, *X. acifer*, *X. filiformis*, and *X. simplicissima* are distributed in different clades in the phylogenetic tree. *Xylaria maitlandii* (Dennis) D. Hawksw. has a somewhat similar stromatal shape to *X. acifer*. However, *X. maitlandii* has hairy stromata, which clearly distinguishes it from *X. acifer* (Ju and Hsieh 2023).

**Additional specimen examined.** China • Hainan Province, Diaoluoshan Area of Hainan Tropical Rainforest National Park; 18°43'31"N, 109°52'14"E; elevation 937 m; on fallen leaves, 18 June 2023, Xiaoyan Pan (HAFFR 130). GenBank accession numbers PQ483150 (ITS) and PQ498328 (RPB2).

### *Xylaria tuberculosa* Xiao Y. Pan, sp. nov.

MycoBank No: 856021
Fig. 3

**Holotype.** China • Hainan Province, Diaoluoshan Area of Hainan Tropical Rainforest National Park; 18°43'33"N, 109°52'20"E; elevation 932 m; on fallen leaves, 18 June 2023, Xiaoyan Pan (HAFFR 123). GenBank accession numbers PQ483148 (ITS), PQ498330 (TUB2), and PQ498327 (RPB2).

**Diagnosis.** Differs from *X. acifer*, *X. hedyosmicola*, *X. petchii*, and *X. polysporicola* by having smaller ascospores.

**Etymology.** "*tuberculosa*" refers to the appearance of the stromata surface, which resembles segmented tubercles formed by the clustered perithecia.

**Teleomorph.** ***Stromata*** upright, solitary to scattered, cylindrical, unbranched, 5–10 mm total length; acute sterile apex, 0.1 mm; fertile portion 3–6 mm long × 0.3–1 mm wide, cylindrical, composed of tightly packed perithecia; stipe glabrous, 1.8–5 mm long × 0.2–0.5 mm wide, longitudinally striate, the base slightly swollen; surface roughened, black, with half-exposed to fully exposed perithecial mounds; interior white to creamy; texture soft. ***Perithecia*** spherical, 180–350 µm diam. ***Ostioles*** papillate. ***Asci*** with eight ascospores arranged in uniseriate manner, cylindrical, 70–105 µm total length, spore-bearing part 50–70 µm long × 5–7 µm wide, stipe 25–45 µm long, with a bluing apical ring in Melzer's reagent, tubular to slightly urn-shaped, 1.8–2.6 µm high × 1.2–2.5 µm wide. ***Ascospores*** brown, unicellular, ellipsoid, inequilateral, with narrowly rounded ends, smooth, 7–8(–9) × 3.3–4.3 µm (M = 7.6 × 3.8 µm, N = 40), with straight germ slit slightly shorter than the spore length on the flattened side, and a hyaline sheath visible in 1% SDS, swollen at both ends, forming non-cellular appendages.

**Remarks.** *Xylaria tuberculosa* forms a distinct branch in the phylogenetic tree with *X. acifer*, *X. betulicola*, *X. crinalis*, *X. filiformis*, *X. hedyosmicola*, *and X. simplicissima*, but it is morphologically different from these six species. *Xylaria acifer* has a needle-like stroma with flatter perithecial mounds and larger ascospores (9.3–12 × 3.7–4.7 µm), while *X. betulicola*, *X. crinalis*, *X. filiformis*, *X. hedyosmicola*, *and X. simplicissima* all have stromata with long sterile apices and significantly larger ascospores, measuring (9.5–21.5 × 3.5–8.5 µm). The ascospores of *X. betulicola*, *X. crinalis*, *X. filiformis*, *X. hedyosmicola*, and *X. simplicissima* measure (11.5–)12–14(–15) × 5–6 µm, (14–)15–16.5(–17.5) × (3.5–)4–5(–6) µm, (9.5–)11.5–13.5(–14.5) × (4–)4.5–5.5(–6) µm, (12–)13–

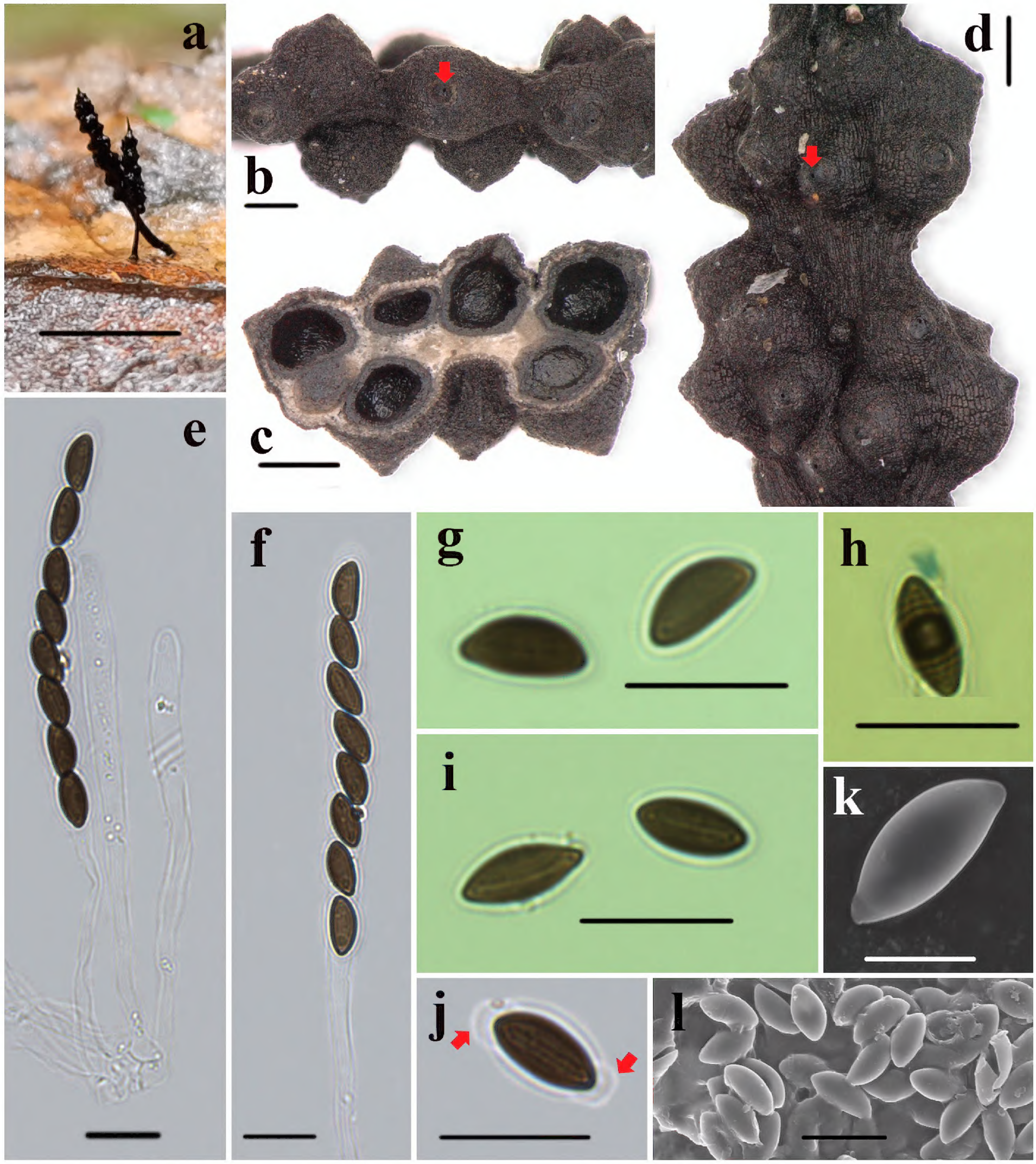


**Figure 3.** *Xylaria tuberculosa* (HAFFR 123) a stromata on leaves **b, d** stromatal surface and ostioles (arrow) **c** section through stroma, showing perithecia **e, f** ascus in 1% SDS **g** ascospores in water **h** ascal apical ring in Melzer's reagent **i** ascospore showing a slightly shorter than spore-length straight germ slit in water **j** ascospore showing non-cellular appendages in 1% SDS (arrows) **k, l** ascospores under SEM. Scale bars: 0.5 cm (**a**); 200 µm (**b–d**); 10 µm (**e–j, l**); 5 µm (**K**).

15(–16.7) × (6–) 6.5–7.5 (–8.5) µm, and (15–)16.5–19(–21.5) × (5–)5.5–6.5(–7.5) µm, respectively (Ma and Li 2018; Pan et al. 2022; Ju and Hsieh 2023).

*Xylaria petchii* Lloyd and *X. polysporicola* Hai X. Ma & X.Y. Pan are similar to *X. tuberculosa* morphologically, as they share cylindrical stromata growing on fallen leaves. Pan et al. (2024) described *X. petchii* from China, noting that this species has a variable fertile portion of the stromata and ascospores measuring

(8.5–)10–12.5(–15) × (4.5–) 5–6.5(–7) µm, which clearly differentiate it from *X. tuberculosa*. *Xylaria polysporicola* differs from *X. tuberculosa* by having a larger apical ring (2.5–4.5 µm high × 2–3.2 µm broad) and larger ascospores ((11.5–)12.5–14.5(–15) × 5.5–8 µm) (Pan et al. 2022). Furthermore, *X. tuberculosa*, *X. petchii*, and *X. polysporicola* are clearly separated in the phylogenetic tree.

*Xylaria himalayensis* Narula & Rawla and *X. meliacearum* Læssøe also resemble *X. tuberculosa* in morphology. *Xylaria himalayensis* can be distinguished by its hairy stipe and larger ascospores ((11.5–)13–15(–15.5) × (4.5–)5–5.5(–6) µm) that lack non-cellular appendages (Ju et al. 2018). *Xylaria meliacearum* can be differentiated by its stromata covered with a sulfur-yellow outer layer and larger ascospores ((19–)21.5–27.5(–31.5) × (5–)5.5–7(–8) µm) (Ju and Hsieh 2023). *Xylaria tentaculata* Ravenel ex Berk. is somewhat similar to *X. tuberculosa* in morphology, but *X. tentaculata* has significantly larger ascospores (20.6–24.4 µm × 8.9–10.8 µm) (Kim et al. 2016).

**Additional specimen examined.** CHINA • Hainan Province, Diaoluoshan Area of Hainan Tropical Rainforest National Park; 18°43'31"N, 109°52'19"E; elevation 929 m; on fallen leaves, 26 February 2023, Xiaoyan Pan (HAFFR 63). GenBank accession numbers PQ483149 (ITS) and PQ498331 (TUB2).

## Discussion

Hainan Tropical Rainforest National Park, located in the central and southern part of Hainan Island (18°33'16"–19°14'16"N, 108°44'32"–110°04'43"E), is the region with the richest forest resources on the island, home to 4,367 species of higher plants (https://www.hntrnp.com/, accessed on 29 October 2024). Symbiosis between fungi and plants is common in nature (Thomas et al. 2016), and the rich plant diversity provides ample ecological niches for fungi, fostering many endemic species. Pan et al. (2022, 2024) described eight *Xylaria* species associated with fallen leaves and petioles. Song et al. (2022) described five new species of *Hypoxylon*, and Deng et al. (2023) published four new species of *Strobilomyces*. Based on morphological characteristics and molecular phylogenetic analysis, this paper describes two new species from Hainan Tropical Rainforest National Park, namely *X. acifer* and *X. tuberculosa*.

To date, 46 *Xylaria* species associated with fallen leaves and petioles have been discovered. Unfortunately, only 18 species' sequences were collected in the National Center for Biotechnology Information, and this study provides sequences for two more species, leaving 26 *Xylaria* species associated with fallen leaves and petioles without genetic sequences. Phylogenetic analysis shows that the two species described in this paper are distinctly separated from other leaf-associated *Xylaria* species. Moreover, a rigorous comparison reveals that the *Xylaria* species lacking molecular sequences are also distinct from the species described in this paper, as reflected in the identification key. The eight *Xylaria* species collected by Pan et al. (2022, 2024) in Hainan grow on highly decomposed leaves. Previous research indicates that fresh leaves and decayed leaves differ significantly in nutrient content (Zeng et al. 2016), suggesting that these fungi play a crucial role in nutrient cycling within ecosystems, a view also supported by Rajtar et al. (2023). Additionally, while Ju and Hsieh (2023) summarized the *Xylaria* species associated with fallen leaves and petioles worldwide, most species' host specificity remains unclear and requires further study.

Below is the identification key for the 46 *Xylaria* species associated with fallen leaves and petioles globally (Pande 1973; Samuels and Rogerson 1990; San Martín et al. 1997, 2001; Huang et al. 2014; Ju et al. 2018; Pan et al. 2022, 2024).

## Key to the species of *Xylaria* associated with fallen leaves and petioles worldwide

1 Stromata surface densely covered by yellow tomentum.....***X. fulvotomentosa***
– Stromata surface lacking yellow tomentum.....**2**
2 Stromata branched, long stipes that bear one to three clavae on each terminal branch .....***X. luxurians***
– Stromata unbranched to occasionally branched.....**3**
3 Fertile parts cylindrical or conical to subglobose, most perithecia gather near the top of the stromata, with several occasionally scattered below.... ***X. petchii***
– Morphology of fertile parts is simpler than above, with perithecia lacking the above cluster patterns .....**4**
4 Stipes glabrous.....**5**
– Stipes tomentose .....**35**
5 Fertile parts filiform.....**6**
– Fertile parts not filiform .....**11**
6 Ascospores with spiral germ slit .....***X. meliacearum***
– Ascospores with straight germ slit .....**7**
7 Ascospores with non-cellular appendages.....**8**
– Ascospores without non-cellular appendages .....**9**
8 Ascospores light brown, short fusoid .....***X. fliformis***
– Ascospores brown to dark brown, ellipsoid.....***X. vagans***
9 Ascospores (15–)16.5–19(–21.5) × (5–)5.5–6.5(–7.5) µm .....
.....***X. simplicissima***
– Ascospores length less than 13.5 µm and width less than 5 µm .....**10**
10 Stromata with an acute apex, ascospores 12–13.5 × 4–5 µm.....***X. eugeniae***
– Stromata with a long acicular apex, ascospores (9–)9.5–10.5(–11) × (3.5–)4–4.5(–5) µm .....***X. vermiformis***
11 Fertile parts cylindrical.....**12**
– Fertile parts not cylindrical .....**27**
12 Ascospores with non-cellular appendages.....**13**
– Ascospores without non-cellular appendages .....**22**
13 Stromata with an outer peeling layer split into narrow or thread-like stripes, ascospores (13.5–)14–15(–17) × (4.5–)5–6(–7) µm.....***X. minuscula***
– Stromata without an outer peeling layer or the outer peeling layer without narrow or thread-like stripes .....**14**
14 Stromata with an outer peeling layer split into band-like stripes, ascospores (8–)8.5–10(–11) × (4–)4.5–5(–5.5) µm.....***X. vittiformis***
– Stromata without an outer peeling layer or the outer peeling layer without band-like stripes .....**15**
15 Stromata surface lacking perithecial mounds or with inconspicuous perithecial mounds .....**16**
– Stromata surface with conspicuous perithecial mounds.....**17**

16 Ascospores (22–)23.5–27(–28) × (8.5–)9–10.5(–11) µm...***X. spiculaticlavata***
– Ascospores 8–10 × 4–6 µm ...***X. kamatii***
17 Ostioles conic-papillate, tilting upwards, ascospores (14–)15–16(–17) × (6.5–)7.0–7.5(–8) µm...***X. appendiculatoides***
– Ostioles papillate or slightly papillate ...**18**
18 Stromata surface blackish brown, ascospores (15–)16.5–18(–19) × (7.5–)8–9(–9.5) µm ...***X. phyllophila***
– Stromata surface black, ascospores width almost less than 7.5 µm.....**19**
19 Stromata with long, filiform apices, much longer than the fertile part ..... ...***X. diaoluoshanensis***
– Stromata with an acute apex, shorter than the fertile part.....**20**
20 Ascospores length greater than 11.5 µm and width greater than 5.5 µm..... ...***X. polysporicola***
– Ascospores length almost less than 11.5 µm and width less than 4.7 µm....**21**
21 Stromata surface with conspicuous to half-exposed perithecial mounds, ascospores (9.3–)10–11(–12) × 3.7–4.7 µm ...***X. acifer***
– Stromata surface with half-exposed to fully exposed perithecial mounds, ascospores 7–8(–9) × 3.3–4.3 µm ...***X. tuberculosa***
22 Stromata with a striped outer peeling layer...**23**
– Stromata without an outer peeling layer or the outer peeling layer without stripes...**25**
23 Stromata with an acuminate or mucronate apex...***X. noduliformis***
– Stromata with a long acicular apex...**24**
24 Stromatal outer peeling layer split into narrow or thread-like stripes, ascospores (8.5–)9–11 × 4–6 µm ...***X. foliicola***
– Stromatal outer peeling layer split into band-like stripes, ascospores (10–)11–12(–12.5) × (5.5–)6–7(–7.5) µm ...***X. vittatipiliformis***
25 Ascospores light brown to brown, (5.5–)6–7 × 3–3.5(–4) µm.....***X. diminuta***
– Ascospores brown to dark brown, length greater than 9 µm and width greater than 4.5 µm...**26**
26 Stromata surface dark vinaceous brown, with slight perithecial mounds..... ...***X. phyllocharis***
– Stromata surface blackish brown, with conspicuous perithecial mounds.... ...***X. neblinensis***
27 Stromata surface lacking perithecial mounds or with inconspicuous perithecial mounds ...**28**
– Stromata surface with conspicuous perithecial mounds...**31**
28 Ascospores broadly ovoid to nearly globose, (11.6–)12.8–16.7(–18) × 8–15 µm...***X. clusiae***
– Ascospores elliptical or nearly semicircular to broadly broad-elliptical, width less than 8 µm ...**29**
29 Ascospores without non-cellular appendages ...***X. delicatula***
– Ascospores with non-cellular appendages...**30**
30 Stromata surface dull grayish brown, ascospores dark brown, (13–)13.5–15(–16.5) × (6–)6.5–7.5(–8 ...***X. hypsipoda***
– Stromata surface dark brown to blackish, ascospores brown, 12–14 × 6–8 µm ...***X. memecyli***

31 Stromata with fertile apices ..... ***X. heloidea***
– Stromata with acute sterile apices ..... **32**
32 Stromata with a long acicular apex, much longer than the fertile part ..... **33**
– Stromata with an acute apex, shorter than the fertile part ..... **34**
33 Stromata 91–147 mm total length, ascospores (10.5–)11.5–13.5(–15) × (5–)5.5–6.5(–7.5) µm ..... ***X. nainitalensis***
– Stromata 3–20 mm total length, ascospores (8.5–)9.5–11(–12) × (4–)4.5–6(–6.5) µm ..... ***X. sicula***
34 Ascospores (12–)12.5–15.5(–17) × (5–)6–7.5(–8) µm ..... ***X. amphithele***
– Ascospores (10–)10.5–12(–12.5) × (5–)5.5–6(–6.5) µm ..... ***X. pisoniae***
35 Ascospores 8–9(–9.5) × 4–4.5(–6.6) µm ..... ***X. imminuta***
– Ascospores length greater than 10 µm ..... **36**
36 Ascospores with non-cellular appendages ..... **37**
– Ascospores without non-cellular appendages ..... **40**
37 Ascospores (21.5–)22.5–24.5(–26) × (6.5–)7–8(–9) µm, with long tubular non-cellular appendages ..... ***X. axifera***
– Ascospores length less than 17 µm, with papillate non-cellular appendages ..... **38**
38 Stromata without a covering of brown tomentum, ascospores (11.5–)12.5–14(–15) × (6–)6.5–7.5(–9) µm ..... ***X. appendiculata***
– Stromata covered with brown tomentum ..... **39**
39 Ascospores (14.5–)15–16.5(–17) × (8–)8.5–9.5(–10) µm ..... ***X. allima***
– Ascospores (10–)10.5–12(–14) × (5–)6–7(–7.5) µm ..... ***X. lima***
40 Fertile parts overlain by dark long spikes ..... ***X. asperata***
– Fertile parts without dark long spikes ..... **41**
41 Stromata surface with half-exposed to fully exposed perithecial mounds .... **42**
– Stromata surface lacking perithecial mounds or with inconspicuous perithecial mounds ..... **43**
42 Fertile parts cylindrical ..... ***X. castilloi***
– Fertile parts filiform ..... ***X. duranii***
43 Fertile parts cylindrical ..... ***X. maitlandii***
– Fertile parts capitate ..... **44**
44 Stromata with rounded apices, ostioles coarsely conic-papillate ..... ***X. aristata* var. *hirsuta***
– Stromata with acute apices, ostioles slightly papillate or papillate ..... **45**
45 Ascospores (10–)10.5–12.5(–14) × (5.5–)6–7(–7.5) µm ..... ***X. aristata* var. *aristata***
– Ascospores (15–)15.5–17(–18) × (6.5–)7.5–9(–9.5) µm ..... ***X. hispidipes***

## Acknowledgements

We thank Kaizhen Lin, Kaili Zhao, Hongdeng Li, and Changzhong Liang for their assistance in the sample collection process.

## Additional information

### Conflict of interest

The authors have declared that no competing interests exist.

## Ethical statement

No ethical statement was reported.

## Funding

This work was supported by the Hainan Provincial Technical Innovation Program for Provincial Research Institutes (No. KYYSLK2024-001) and the Hainan Tropical Rainforest National Park Comprehensive Resource Survey and Monitoring Project (No. ZZZB2024-11-1).

## Author contributions

Zongzhu Chen and Yiqing Chen designed the study; Xiaoyan Pan collected the specimens; Tingtian Wu and Yuanling Li conducted the molecular experiments; Jinrui Lei and Xiaohua Chen performed the morphological observations; Xiaoyan Pan, Zongzhu Chen, and Yiqing Chen organized the data and conducted the phylogenetic analysis; Xiaoyan Pan wrote the manuscript; Xiaoyan Pan revised and polished the article. All authors have read and agreed to the published version of the manuscript.

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## Data availability

Datasets analyzed in this study are publicly available. All the gene sequences obtained in this article can be searched in GenBank (https://www.ncbi.nlm.nih.gov/genbank/; Table 1). All new taxa are saved in MycoBank (https://www.mycobank.org/).

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